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Rat Skin Main Neutral Protease: Immunohistochemical Localization

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Specific antiserum against the purified rat skin main neutral protease was used in double layer immunofluorescent method to localize the enzyme in normal rat skin. The specific immunofluorescence was seen in dermal cells that were identified as mast cells on basis of their metachromatic granules. Enzyme histochemical staining with naphthol AS-D chloroacetate localized to the same cells that exhibited specific immunofluorescence. The granules of isolated rat mast cells also gave a specific reaction with the immunohistochemical technique.

The results provide further evidence for the suggestion that rat skin main neutral protease is identical with the rat mast cell "chymase."

A chymotrypsin-like protease representing the main proteolytic activity of rat skin at neutral pH was purified recently by Seppä and Järvinen [1]. The biochemical properties of the neutral protease closely resemble those of the rat mast cell "chymase." Treatment of rats with the mast cell degranulator, compound 48/80, sharply decreases the amount of active neutral protease in the skin, which speaks for a mast cell origin of the enzyme.

The results of immunohistochemical localization of rat skin main neutral protease are presented in this study and these support the suggestion that the protease is a mast cell enzyme.

MATERIALS AND METHODS

Preparation of Tissue

Adult male and female rats of Long-Evans and Wistar strains were used. Rats were killed using ether, and the hair was clipped and shaved from the dorsum. Some of the rats were depilated with a commercial depilating cream, Veet, which was washed away after the treatment. Pieces of skin, including the subjacent panniculus, measuring approximately 10 × 5 mm were cut, and were then rapidly submerged in liquid nitrogen. The pieces were stored in small airtight plastic containers at −70°C, until used.

Preparation of Skin Extracts

The treatment of rats with compound 48/80 was carried out as described earlier [1]; the rats were injected intraperitoneally with compound 48/80 dissolved in saline to a concentration of 1 gm/liter, the dose was 0.1 mg/kg on the first day and was then increased daily, being 0.5 mg/kg on the 5th day. The rats were killed on the 6th day and the skins obtained and processed, as for normal rat skin, as described earlier [1]. Briefly, the skin is first homogenized and extracted in 20 mM sodium phosphate buffer, pH 8.0; this extract is called B-extract. The sediment after centrifuging is then rehomogenized and extracted in 20 mM sodium phosphate, 2 M KCl, pH 8.0, and the extract dialyzed against 10 mM potassium phosphate, pH 6.0. The enzyme is precipitated in the dialysis and is redissolved in 20 mM sodium phosphate, 2 M KCl, pH 8.0; this extract is called B-Sdp extract.

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Abbreviations:
PBS: phosphate-buffered saline
FITC: fluorescein isothiocyanate

Isolation of Rat Mast Cells

The cells of 2 male Long-Evans rats were collected by lavage of the peritoneal and thoracic cavities of each rat with 20 ml of Tyrode's solution containing 0.1% (w/v) gelatin (E. Merck AG) and 50 μg/ml commercial heparin (Sigma). Mast cells were isolated as described by Yurt and Austen [2] by centrifuging through 22.5% (w/v) Metrizamide (Nyegaard & Co, Oslo). The cells from the pellet were then suspended in Tyrode's solution containing 0.1% (w/v) gelatin and 10 μg/ml deoxyribonuclease (Sigma), to a concentration of 5×10^5 cells/ml, and 0.2 ml aliquots were used for preparing histological preparations by centrifuging at 150 ×g for 15 min in a cytocentrifuge. The slides were then postfixed in 96% ethanol for 15 min and processed for immunohistochemistry or histochemistry as described later.

Preparation of Antiserum

The rat skin neutral protease was purified starting from the B-Sdp extract, by chromatography in Sephadex G-200 and hydroxyapatite, as described earlier [1]. The enzyme preparation gave one homogenous band in sodium dodecylsulfate gel electrophoresis. The purified enzyme was concentrated by ultrafiltration to a ⅓ using a Diaflo PM-10 membrane, KCl was added to the preparation before concentrating to keep the enzyme in solution during the process. The protein concentration of the preparation was 75 μg/ml, assayed using the method of Lowry et al [3]. The concentrated enzyme was then dialyzed against PBS, and divided into 5 aliquots, 1 ml each. A white male albino rabbit was injected with a mixture of 1 ml of the enzyme preparation and 1 ml of Freund's complete adjuvant. The injections were given intradermally in the scapular area. A control sample of serum (preimmune serum) was obtained before immunization. The injections were repeated 4 times at 2-week intervals. Two weeks after the last injection 45 ml of blood was taken, and the serum was used.

Preparation of the Gammaglobulin Fraction

The preparation of the gammaglobulin fraction of the antiserum was done by precipitation with ammonium sulfate according to a modification of the method of Kendall [4]. The prepared gammaglobulin fraction was analyzed by cellulose acetate electrophoresis.

Immunodiffusions and Immunoelectrophoreses

Double immunodiffusion was performed on plates of 1% (w/v) agarose, the medium was 1 M NaCl which was necessary to keep the enzyme from adsorbing to the gel. Sodium azide was added to a concentration of 0.05% (w/v) to inhibit microbial growth. The reaction was carried out at room temperature in a humid chamber for 1–5 days. The gels were then pressed, and washed in 1 M NaCl [4]. This was repeated twice, and the gels were then washed in physiological saline at least 5 times, and then finally twice in distilled water and were pressed between each washing. The gels were dried using a hairdrier, and stained with Coomassie Brilliant Blue R 250 [4].

Immunoelectrophoresis was performed according to the method of Svendsen as described in [4]. 1% (w/v) agarose in sodium barbital-glycine-Tris, pH 8.8, I=0.08, with 0.5% (w/v) Triton X-100 and 0.05% (w/v) NaN_3 added, was used for the gels. The samples were dialyzed against the buffer prior to application to the gel in order to remove the high concentrations of salt. The electrophoresis was carried out at a voltage of 2 v/cm for 3 hr, and the gels were subsequently developed with the antiserum.

Immunohistochemical Staining

Immunohistochemical staining was performed using the double layer immunofluorescence technique, in which rabbit antienzyme antibody is first bound to the tissue and then treated with goat antirabbit immunoglobulin labeled with fluorescein isothiocyanate (FITC). Frozen sections were fixed for 15 min in 96% ethanol, washed for 15 min in phosphate-buffered saline (PBS), and then incubated 15 min with

bovine immunoglobulin, 5 mg/ml in PBS, to counteract nonspecific staining, and after a wash in PBS, with dilutions of the antiserum, control serum, or PBS. The reaction was carried out in a humid chamber at room temperature for 30 min. Following a 15 min wash in PBS, the sections were treated with FITC-conjugated goat antirabbit immunoglobulin (Nordic Immunology, batch 27-175) for 30 min, washed, and mounted in a 9:1 mixture of glycerin and buffered saline.

The sections were examined with a Leitz Orthoplan fluorescence microscope with a Ploem vertical illuminator under incident ultraviolet light (activation filters were BG 12 and BG 38, and barrier filter was K 510) with a mercury vapor lamp as the light source. Leitz Orthomat camera with Agfapan 400 film was used for the microphotographs.

Controls for Immunohistochemical Specificity

The evaluation and identification of nonspecific fluorescence were accomplished by substituting control serum for specific antiserum. Preimmune serum from the immunized rabbit was used routinely as control serum. In addition, specifity was ascertained by substituting the antiserum with absorbed antiglobulin. This was prepared from the immunoglobulin fraction of the antiserum by titrating with the purified rat skin neutral protease until precipitation ceased and removing the formed precipitate by centrifuging. Dilutions of the absorbed antiglobulin were used immediately in the immunohistochemical reaction and the results were compared with the reaction obtained with the unabsorbed specific immunoglobulin.

Histochemical Staining

Naphthol AS-D chloroacetate, a histochemical substrate for proteinases, was used as substrate in the enzyme histochemical reaction on sections of rat skin, postfixed in 96% ethanol, or on sections that had just previously been stained using the immunohistochemical method. The incubation medium was prepared as described by Starkey and Barrett [6]: 0.1 M potassium phosphate, pH 6.0, freshly prepared and filtered (2 mg/ml) Fast Garnet GBC (Sigma Chemical Company), and (5 mg/ml) naphthol AS-D chloroacetate (Sigma Chemical Company) in dimethylsulfoxide, were mixed in the proportion of 60:20:1, and filtered before use. The sections were incubated for 5–60 min for assessing the optimal time of incubation; it was observed that times in excess of 30 min did not add to the specific staining, and thus 30 min was used routinely. Nonenzymatic staining was controlled by incubating sections in media that contained 2 mM phenylmethylsulfonylfluoride (Schwarz/Mann). After incubation the sections were washed in distilled water and mounted. Agfapan 25 film was used for photography with Leitz Orthomat microscope camera.

Staining with toluidine blue was carried out in an aqueous *0.1%* (w/v) solution of the dye, as previously described [7].

RESULTS

Specificity of Antiserum

The serum obtained before immunization did not produce precipitates in double immunodiffusion. A single line of precipitin was observed when the antiserum was reacted against the purified protease of rat skin, B-Sdp extract of normal rat skin, and also a weak reaction against B-Sdp extract of 48/80 treated rats' skin (Fig 1*A*). No precipitate was observed, even after staining, when the antiserum was reacted against B-extract of normal rat skin, rat plasma or B-extract of 48/80 treated rats' skin (Fig 1*A*). The antiserum was also tested against the neighboring fractions from the last stage of purification, hydroxyapatite chromatography [1], as they would contain the substances that would be the most probable contaminants, and no precipitates were observed. In another experiment the precipitin lines were seen to be confluent, which indicates identity of the antigen in the different samples.

The skin extracts, rat plasma and the purified protease were subjected to immunoelectrophoresis in agarose gel that contained 0.5% (w/v) Triton X-100. The samples were dialyzed against the buffer of electrophoresis gels to remove the excess of salt. The purified protease was the only sample that produced a precipitate, a single arc on the cathode side of the application well (Fig 1*B*).

Immunohistochemical Results

In the sections of rat skin which were incubated first with the antiserum and then with the fluorescein conjugate of goat antirabbit immunoglobulin, the specific fluorescence was localized to solitary cells that have a characteristic distribution. The majority of the cells were seen to be along the subdermal vessels (Fig 2*A*), another row of cells was seen just beneath the epidermis (Fig 3*A*) and, in addition, a few scattered cells were observed in the dermis. At higher magnification the specific fluorescence was seen to be located in granules of the cells (Fig 4*A*, 5*A*). Bright yellowish autofluorescence was observed in the hairs and the keratin layer of the epidermis (Fig 3*A*). Nonspecific staining was observed in the dermis in a fibrillar pattern, and diffusely in the lower layers of the epidermis. The specific fluorescence decreased when the antiserum was diluted, a dilution of 1/140 still gave a reaction, but a dilution of 1/700 was negative. The reaction was inhibited by substituting the antiserum in the first layer with absorbed antiglobulin. No reaction

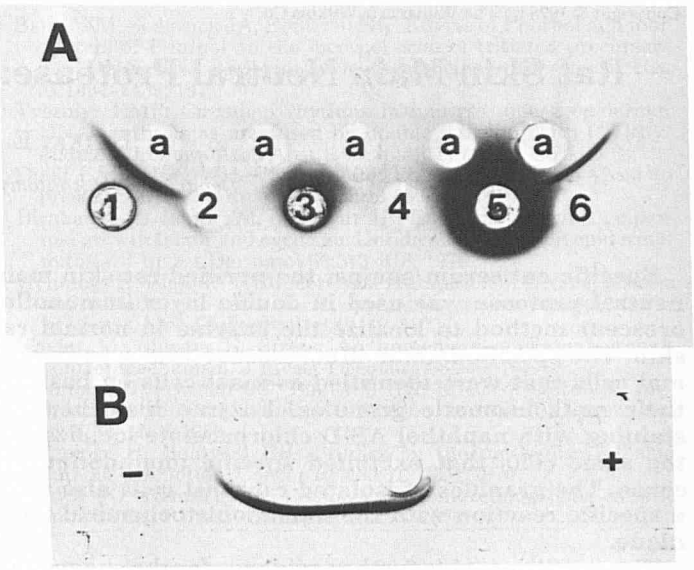

FIG 1. *A*, Double immunodiffusion in agarose gel of the anti-rat skin main neutral protease-serum in the presence of 1 M NaCl against: *1* = B-Sdp extract of normal rat skin, *2* = B-extract of normal rat skin, *3* = B-Sdp extract of 48/80 treated rats' skin, *4* = B-extract of 48/80 treated rats' skin, *5* = rat plasma, and *6* = purified rat skin neutral protease. *B*, Immunoelectrophoresis of purified rat skin neutral protease.

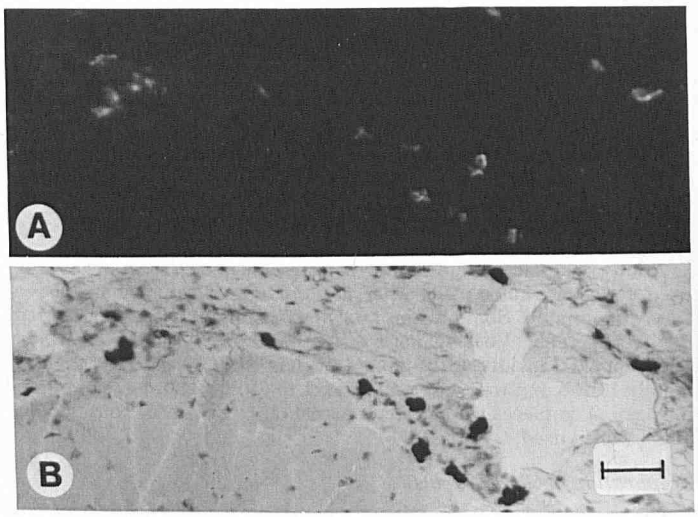

FIG 2. *A*, Fluorescent staining of rat skin section by antiserum against rat skin main neutral protease. *B*, The same section after toluidine blue staining. Dermis is in the upper part and muscle layer in the lower part of the photograph. The specific immunofluorescence is seen in cells that show metachromasia and pattern of distribution characteristic of mast cells. *Scale bar* 50 μm.

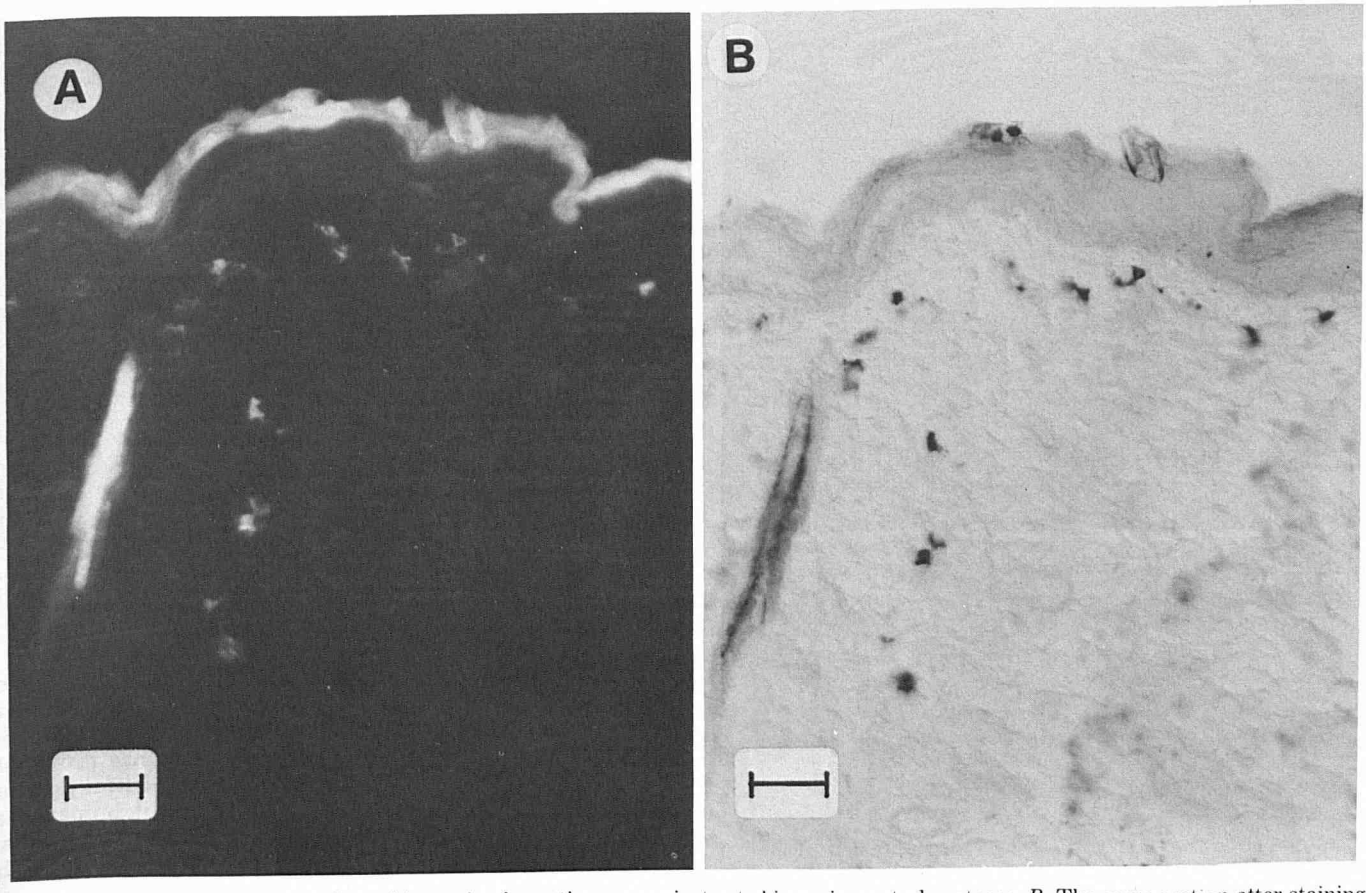

FIG 3. *A*, Fluorescent staining of rat skin section by antiserum against rat skin main neutral protease. *B*, The same section after staining by naphthol AS-D chloroacetate reaction. The specific immunofluorescent staining is seen in solitary cells beneath epidermis and deeper in dermis that show a pattern of distribution characteristic of mast cells. The same cells also stain by naphthol AS-D chloroacetate reaction. The keratin layer of epidermis, and hair show autofluorescence, nonspecific staining is seen in the lower layers of epidermis and, as a fibrillar background staining in dermis. *Scale bar* 50 μm.

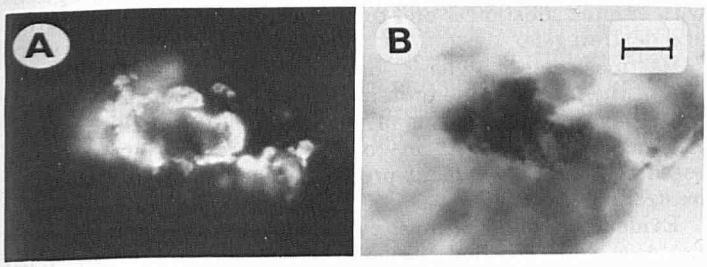

FIG 4. *A*, Fluorescent staining of rat skin section by antiserum against rat skin main neutral protease. *B*, The same section after toluidine blue staining. The specific immunofluorescence is seen in the granules of a dermal cell. Stained with toluidine blue the granules show metachromasia typical for mast cells. *Scale bar* 10 μm.

was obtained when the antiserum was replaced with normal rabbit serum or PBS.

The immunohistochemical reaction was also performed on isolated peritoneal mast cells. The specific fluorescence was seen in the granules of the cells (Fig 6).

Histochemical Results

After the immunohistochemical staining the sections were stained using either toluidine blue or the enzyme histochemical reaction with naphthol AS-D chloroacetate. The cells that showed specific immunofluorescence were seen to stain metachromatically with toluidine blue (Fig 2*B*), and at higher magnification the metachromasia was seen to reside in the granules of the cells (Fig 4*B*). The reaction with naphthol AS-D chloroacetate also localized to the granules of the cells that showed specific immunofluorescence (Fig 5*B*).

DISCUSSION

A common problem in enzyme histochemistry is that many enzymes are dissolved in the incubation media, resulting in distorted histochemical localization. The neutral protease of rat skin is poorly soluble in dilute salt solutions and it is possible to localize it in unfixed tissue sections. Ethanol postfixation was used to preserve the histological details.

The first guarantee of the specificity of the antiserum is the homogeneity of the antigen. The antiserum in the present study was produced with the purified neutral protease of rat skin which was homogenous in sodium dodecyl sulfate gel electrophoresis [1]. Further evidence of the homogeneity of the purified enzyme was obtained in immunoelectrophoresis, where the antibody gave only one arc of precipitin.

The specificity of the antibody was assessed using double immunodiffusion in agarose containing 1 M NaCl. The high concentration of salt was necessary to keep the enzyme from adsorbing to the gel. If the immunodiffusion was performed in the routine physiological saline media, precipitates were obtained just adjacent to the sample wells. The results of the double immunodiffusions indicate specificity of the antiserum: preimmune serum produces no precipitate, the antiserum produces a single line of precipitate with the purified protease and with skin extracts that contain biochemically measurable enzyme activity, and the precipitin lines observed exhibit a reaction of identity, whereas the antiserum does not produce precipitates with skin extracts that do not contain the enzymatic activity.

Specific immunofluorescence was observed in cells that were localized in rows along subdermal vessels and beneath the epidermis; scattered cells were also seen elsewhere in the dermis. At higher magnification the fluorescence was seen in the

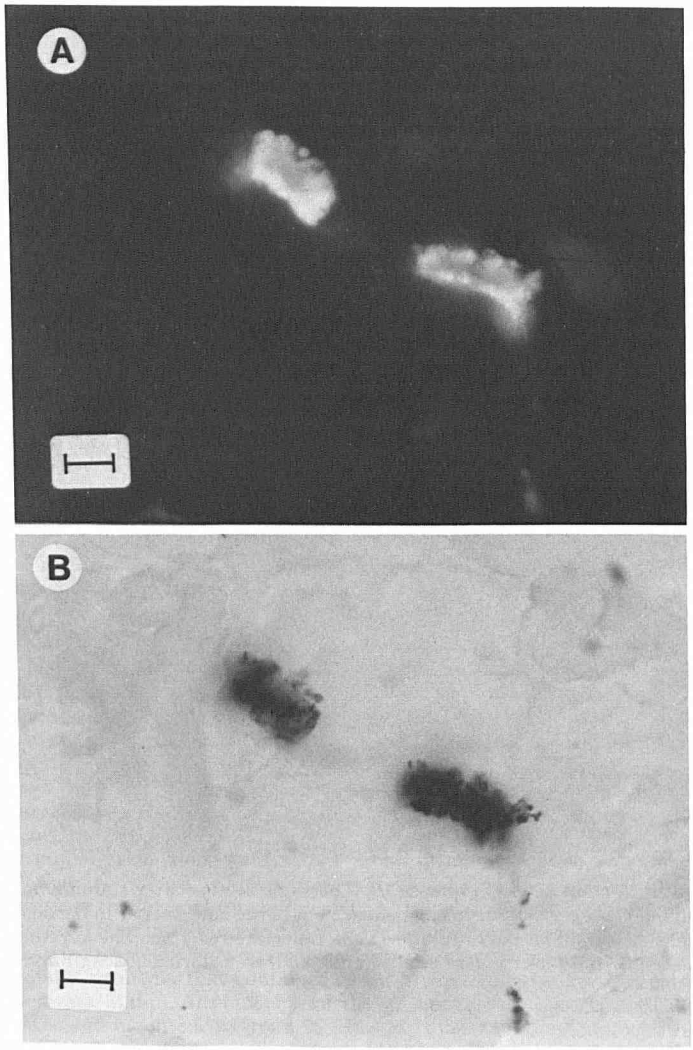

FIG 5. *A*, Fluorescent staining of rat dermis by antiserum against rat skin main neutral protease. *B*, The same section after staining by naphthol AS-D chloroacetate reaction. Staining by both methods is localized to the granules. *Scale bar* 10 μm.

granules of the cells. The granules stained metachromatically with toluidine blue. This establishes the identity of the cells as mast cells according to the definitions given by Montagna [8] and Selye [9a]. Basophil polymorphonuclear leucocytes were not observed in the sections studied.

The granules of isolated rat mast cells also reacted specifically when stained with the immunohistochemical technique. The staining localized to the surface of the granules. This is either due to steric hindrance to binding of the antibody, or due to factual localization of the enzyme in the granules.

The specificity of the immunohistochemical localization was controlled using procedures commonly recommended [10,11]. The localization fullfills the demands of these criteria: the reaction was not obtained with preimmune serum or with the antienzyme gammaglobulin that had been depleted of the antibody by treatment with the antigen, (absorbed antiglobulin) and the intensity of the reaction decreased when the antiserum was diluted.

The mast cells are known to be autofluorescent [10]. In addition, they are prone to nonspecific staining, because they tend to adsorb protein. Nonspecific staining was counteracted by treating the sections with bovine immunoglobulin prior to the antiserum in order to block the sites for nonspecific adsorption. The autofluorescence was insignificant when ethanol was used for fixation.

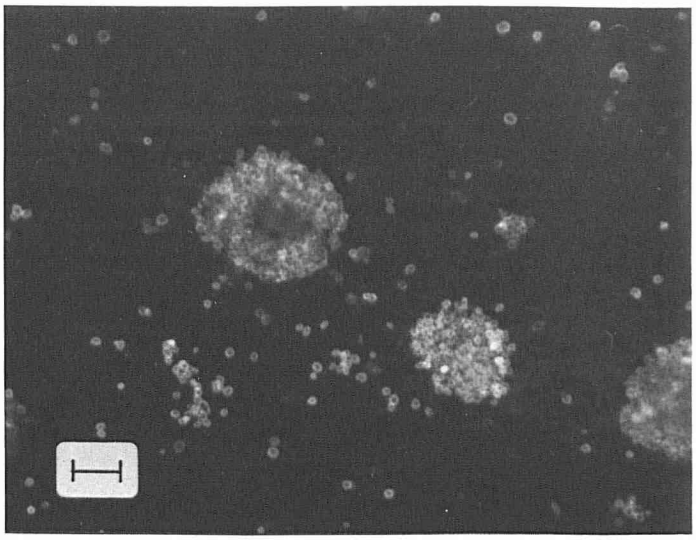

FIG 6. Fluorescent staining of isolated peritoneal and pleural mast cells by antiserum against rat skin main neutral protease. The specific immunofluorescence is localized to the granules. *Scale bar* 10 μm.

The immunohistochemically observed localization of the enzyme was further confirmed by the exactly coinciding localization of the enzyme histochemical staining with naphthol AS-D chloroacetate, a histochemical substrate that has previously been shown to be hydrolyzed by the purified neutral protease of rat skin [1]. The hydrolysis of this substrate in human mast cells has been demonstrated previously [12]. The hydrolysis of a nearly related compound, naphthol AS chloroacetate, has been localized to rat mast cells [13].

The similarities between the reported properties of the rat mast cell "chymase" and the properties of the purified neutral protease of rat skin were pointed out previously [1]: both enzymes preferably hydrolyze esters of aromatic amino acids (i.e., they are "chymotrypsinlike"), are inhibited by serine protease inhibitors, are highly cationic, tend to form aggregates with anionic substances and to be adsorbed by polyacrylamide and dextran gels.

Rat mast cell "chymase" has been estimated to represent 20% of the total granule protein [14], and mast cells are known to be plentiful in the skins of mammals [9b]. Rat skin can be assumed to contain a plenty of "chymase." The purified protease represents the main proteolytic activity of rat skin at neutral pH [1].

Evidence in favor of mast cell origin of the rat skin neutral protease was obtained previously from an experiment with compound 48/80 [1], a mast cell degranulator that causes disappearance of mast cells from the subcutanous tissue [15]. Treatment of rats with compound 48/80 for 5 days caused a sharp decrease in the amount of neutral protease in rat skin [1].

In this study, rat skin main neutral protease has been localized to the mast cells of normal rat skin using immunohistochemical technique. Specific immunofluorescence was also detected in isolated rat mast cells. The results provide further evidence on the suggested identity of the purified rat skin main neutral protease with "chymase," the chymotrypsin-like protease of rat mast cells [2,16].

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Announcement

The Foundation for Research in Dermatology will sponsor an intensive 2-day course entitled "Histologic Diagnosis of Inflammatory Skin Diseases: A Method by Pattern Analysis" to be given by Dr. A. Bernard Ackerman, Professor of Dermatology and Pathology and Director of Dermatopathology, New York University School of Medicine, at the Catholic University in Rome, Italy, on Friday and Saturday, April 20 and 21, 1979. Enrollment is limited, and enquiries should be directed to Dr. Ferdinando Serri, Professor and Chairman, Dept. of Dermatology, Università Cattolica, Largo Gemelli 8, Rome, Italy.